IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Darrell Sleep
Application No. 10/522,074
U.S. National Stage of PCT
Application No. PCT/GB03/03273
Filed July 8, 2005

Examiner: S. Gudibande
Group Art Unit: 1654
Confirmation No. 7594

GENE AND POLYPEPTIDE SEQUENCES

(Atty. Docket No. P30,358 USA)

DECLARATION OF DARRELL SLEEP UNDER 37 C.F.R. § 1.132

I, Darrell Sleep, hereby declare the following:

1. I am an inventor named on the above-identified application (hereafter "the present application").

2. I am presently Senior Manager of Novozymes R&D UK, at Novozymes Biopharma UK Limited (formerly Delta Biotechnology Limited), the assignee of the present application.

3. I have read the present application and am familiar with the Examiner's arguments, in the Office Action dated January 9, 2008.

4. The studies discussed below were conducted under my supervision and direction. I am familiar with the procedures used and the data obtained through the studies.

5. An extensive study was conducted to determine the effect of various conservative substitutions of the FIVSI motif (as defined by Claim 1) at each of its positions on the ability of the motif within the context of a leader sequence to enhance expression of a recombinant heterologous protein (the protein chosen to illustrate this was recombinant, human albumin; "rHA"). In addition, the effect of repositioning the motif within the leader

sequence has been tested. The enclosed data demonstrate that claimed variants retain the beneficial effect of the FIVSI motif.

6. Yeast strains containing a plasmid with a *modified* FIVSI motif (i.e. a conservative variant of FIVSI) within the leader sequence were constructed and analysed as follows. Via a two step cloning strategy the ~0.12kb *Bfr*I-*Bgl*II fragment located at the 5' end of the rHA cDNA comprising the 3' end of the *PRB1* promoter and most of the modified HSA/*MFα*-1 fusion leader sequence was replaced with a double stranded oligonucleotide linker encoding a modified FIVSI motif. The sequences of the motifs and their positions within the leader sequence are indicated in the table below.

Motif	Protein sequence (-24 to -1 or -25 to -1)	Position of Motif
FIVSI	MKWVFIVSILFLFSSAYSRSLDKR	-20 to -16
YIVSI	MKWVYIVSILFLFSSAYSRSLDKR	-20 to -16
FVVSI	MKWVFVVSILFLFSSAYSRSLDKR	-20 to -16
FMVSI	MKWVFMVSILFLFSSAYSRSLDKR	-20 to -16
FILSI	MKWVFILSILFLFSSAYSRSLDKR	-20 to -16
FIVTI	MKWVFIVTILFLFSSAYSRSLDKR	-20 to -16
FIVSV	MKWVFIVSVLFLFSSAYSRSLDKR	-20 to -16
FIVSM	MKWVFIVSMLFLFSSAYSRSLDKR	-20 to -16
FIVSA	MKWVFIVSALFLFSSAYSRSLDKR	-20 to -16
FIVSI+I	MKWVFIVSILFLIFSSAYSRSLDKR	-21 to -17
IIVSI+I	MKWVIIVSILFLIFSSAYSRSLDKR	-21 to -17

7. A yeast host cell was transformed to leucine prototrophy with the control plasmids pAYE443 and pAYE646. Plasmid pAYE646 comprises an expression construct as exemplified in the present application, i.e. including FIVSI in the leader pre sequence.

Plasmid pAYE443 comprises an expression construct encoding the same protein, in which the leader pre sequence includes the motif SFISL as disclosed in the prior art. Figure 22 of the present application provides further details of these plasmids. A series of pSAC35 disintegration vectors were prepared with the variant leader pre sequences defined above and, likewise, used to transform a yeast host cell to leucine prototrophy. The rHA productivities of the transformed yeasts were assessed in YEPD shake flask culture, 30°C, 200rpm for 4 days, by rocket immunoelectrophoresis of the culture supernatant against an rHA standard curve ($mg.L^{-1}$). The results of this analysis are presented in Figures 1 and 2.

8. As can be seen from Figures 1 and 2, where the leader sequence responsible for secretion of rHA contained FIVSI (i.e. plasmid pAYE646), production of rHA was greater than that from the control yeast strain in which the leader sequence contained the prior art motif SFISL (i.e. plasmid pAYE443). It can also be seen that motifs containing conservative substitutions of the first, second, third, fourth or fifth positions of the motif FIVSI provided enhanced rHA production, in the same way as FIVSI, compared to the prior art sequence of SFISL.

9. Figure 2 shows that the precise location of the pentapeptide motif within the pre sequence is not essential for the beneficial effect. Where the FIVSI motif occupies positions –17 to –21 of the leader sequence as a result of the introduction of an additional isoleucine residue within the leader sequence (i.e. "FIVSI+I"), compared to the –16 to –20 position as exemplified in Figure 1 of the description, high level rHA production is retained.

10. Results from Figure 2 comparing the rHA production of FIVSI at –17 to –21 and IIVSI at –17 to –21, show that the latter motif, which contains a non-conservative substitution at position 1, i.e. a substitution that is *outside of the claimed motif*, is *less effective* at promoting high level rHA production. In fact, not only does the non-conservative

substitution result in a motif that is less effective than FIVSI, it also produces a motif that is less effective than the wild-type sequence SFISL.

11. In another study, a test plasmid pDB3205 was constructed to contain a *Not*I expression cassette including the *PRB1* promoter, a modified HSA pre sequence including the FIVSI motif, a non-codon optimised synthetic transferrin (S415A, T613A) gene and a modified *ADH1* terminator. The FIVSI motif was positioned at -14 to -10 relative to the C-terminal end of the HSA pre sequence. A control plasmid pDB3605 was constructed to be identical to pDB3205 with the exception that it contains a HSA pre sequence in place of the modified HSA pre sequence. Where pDB3205 contains DNA encoding the motif FIVSI, pDB3605 contains DNA encoding SFISL.

12. A further test plasmid pDB3606 was constructed to contain a *Not*I expression cassette including the *PRB1* promoter, a modified invertase pre sequence including the FIVSI motif, a non-codon optimised synthetic transferrin (S415A, T613A) gene and a modified *ADH1* terminator. The FIVSI motif was positioned at -13 to -9 relative to the C-terminal end of the invertase pre sequence. A control plasmid pDB3221 was constructed to be identical to pDB3606 with the exception that it contains the native invertase pre sequence in place of the modified invertase pre sequence. Where pDB3606 contains DNA encoding the motif FIVSI, pDB3221 contains DNA encoding LFLLA.

13. The sequences of the pre sequences encoded within the expression cassettes of the four plasmids are indicated below. The motif FIVSI is underlined.

Plasmid	Pre sequence	Protein sequence of pre sequence
pDB3205	Modified HSA pre	MKWVFIVSILFLFSSAYS
pDB3605	HSA pre	MKWVSFISLLFLFSSAYS
pDB3606	Modified Invertase pre	MLLQAFFIVSIGFAAKISA
pDB3221	Invertase pre	MLLQAFLFLLAGFAAKISA

14. Each of the above plasmids was used to transform a yeast host cell to leucine prototrophy. The recombinant transferrin (rTF) productivities of the transformed yeasts were assessed in YEPD shake flask culture, 30°C, 200 rpm for 4 days, by rocket immunoelectrophoresis of the culture supernatant against an rTF standard curve. The results of this analysis are presented in Figure 3.

15. As can be seen from Figure 3, where the HSA leader sequence responsible for secretion of rTF contained FIVSI (i.e. plasmid pDB3205), production of rTF was greater than that from the control yeast strain in which the HSA leader sequence contained the prior art motif SFISL (i.e. plasmid pDB3605). Similarly, where the invertase leader sequence responsible for secretion of rTF contained FIVSI (i.e. plasmid pDB3606), production of rTF was greater than that from the control yeast strain in which the invertase leader sequence contained the prior art motif SFISL (i.e. plasmid pDB3221).

16. The data show that the beneficial effect of including FIVSI in a leader sequence is not limited to improving secretion of albumin as a desired protein, but extends to improving secretion of other proteins, such as transferrin, as a desired protein. Furthermore, the beneficial effect is not limited to improving secretion of a desired protein in the context of a modified albumin secretion pre sequence, but extends to improving secretion in the context of other pre sequences, such as a modified invertase pre sequence.

I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 24th June 2008

Darrell Sleep

Darrell Sleep

Figure 1

Rocket Immunoelectrophoresis results

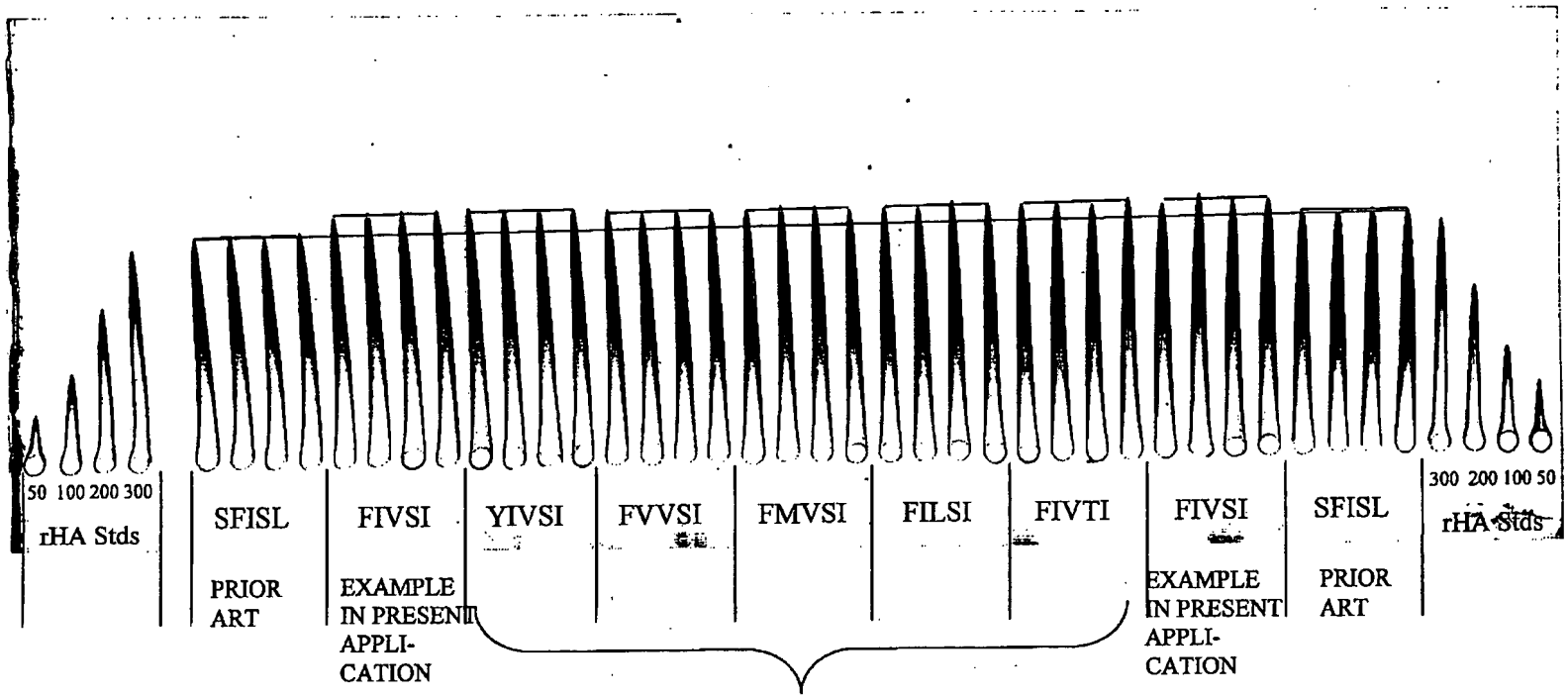

Figure 2

Rocket Immunoelectrophoresis results

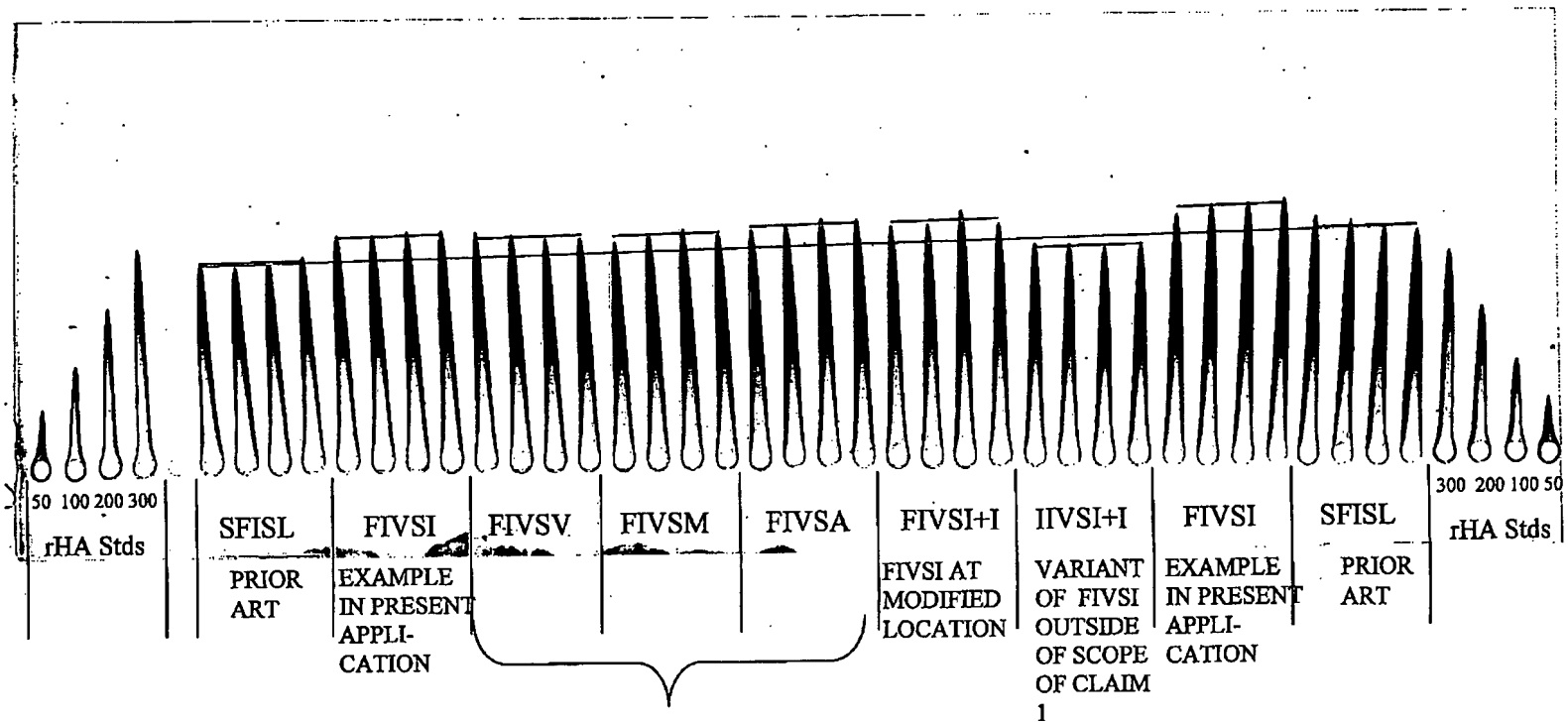

Figure 3

Immunoelectrophoresis results